MALONATE AND CARROT ROOT RESPIRATION

By VERA F. HANLY,* K. S. ROWAN,* and J. S. TURNER*

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Summary

Following a review of earlier work with malonate as an enzyme and respiration inhibitor, direct evidence is provided of the existence in carrot root tissue of cytochrome oxidase and succinic dehydrogenase (S.D.). Malonate is clearly effective as an inhibitor of carrot root respiration only at low pH. Its effects at higher pH are, however, fully described and discussed. It is postulated that in this tissue a significant part of the respiration is mediated by enzyme systems not inhibited by malonate, KCN, or CO; that the remainder, whose activity is varied by wounding and aging, and by ionic exchange and uptake, involves an organic acid cycle of the Krebs type. The effects at low pH and low concentration of malonate (0.005-0.02M) may be explained as due to inhibition of succinic dehydrogenase only; under these conditions self reversal of inhibition, and reversal by addition of succinate, are both possible. At higher concentrations (0.04-0.05M) and low pH, malonate is assumed to inhibit not only S.D. but other enzymes concerned in pyruvate oxidation; this explains the lack of self reversal, lack of reversal by added succinate, and the failure to demonstrate accumulation of succinate in poisoned tissue; under these conditions, when inhibition is to the basal level, the R.Q. is high, presumably because pyruvate is diverted to form fermentation products.

I. PREVIOUS WORK

(*a*) *Malonate as an Enzyme Inhibitor*

Malonate has been generally assumed to inhibit succinic dehydrogenase (S.D.) specifically and it has therefore been widely used in the demonstration of the existence of an organic acid cycle in respiration.

Its action on respiration was discovered by Thunberg (1909). Later, Quastel and Wheatley (1925) noted that the rapid dehydrogenation of succinic acid by resting bacteria, in anaerobiosis, was greatly retarded in the presence of malonate. They used a medium buffered at pH 7.4 and succinate was at one-tenth the concentration of malonate. Substituted acids, such as ethyl- or hydroxymalonic acids were inactive.

In 1931 Quastel and Wheatley stressed the importance of malonate in respiration studies and showed that it inhibited the oxidation of succinate by muscle and brain tissue as well as by bacteria. The oxidation of fumarate or malate was not affected by malonate and this provided evidence against the view that fumarate might be oxidized on the succinic enzyme system. More-

* Botany School, University of Melbourne.

over, malonate did not prevent *p*-phenylenediamine oxidation by tissue, thus the dehydrogenase end of the aerobic oxidation system was taken to be the site of inactivation by malonate.

In 1938 Hopkins, Morgan, and Lutwak Mann provided clear evidence to show that succinic dehydrogenase does not function without intact —SH groups. The enzyme was inactivated by glutathione (GSSG), which oxidizes these groups; after the GSSG had been removed, the activity of the enzyme was restored by the addition of reduced glutathione. It was then found that malonate can protect the enzyme system against the action of GSSG. This observation was confirmed by Potter and Dubois (1943), who also found that malonate protects the enzyme against inactivation by diverse —SH reagents, such as quininoid compounds and heavy metals.

In these experiments (Hopkins, Morgan, and Lutwak Mann 1938) the pH was 7.4; enzymic activity was measured by the rate of reduction of methylene blue or of cytochrome *c*. Malonate inhibition was reversible and the enzyme system, incubated with both GSSG and malonate, was found to have lost none of its original activity after the two inhibitors had been washed away. The protection was afforded by malonate at concentrations much lower than those necessary to inhibit the enzyme activity.

Malonate apparently establishes no special relationships with thiol compounds, so the protection must be indirect. Hopkins, Morgan, and Lutwak Mann (1938), Krebs and Eggleston (1940), and Potter and Dubois (1943) all agree that the inhibition of succinic dehydrogenase by malonate is competitive, as the degree of inhibition depends on the ratio of succinate to malonate present. Potter and Dubois showed that a purified enzyme had a much greater affinity (50/1) for malonate than it had for its normal substrate, succinate. Potter and Elvehjem (1937) have demonstrated inhibition of the succinic enzyme by malonic and oxalic acids and to a lesser extent by adipic and glutaric acids; they agree with the above authors in ascribing the action of these inhibitors to their possession of two —COOH groups, which, for high activity should be in close proximity in the inhibitor molecule. The pH optimum for succinic dehydrogenase is between 7 and 8, and both substrate and inhibitors may be considered to combine with the enzyme as ions. Potter and Dubois have put forward the view that the ions of succinate and malonate compete on the enzyme surface for the same two centres of activity, to which the —COOH groups become attached; the —SH group is supposed to lie between these centres and hence is shielded when the malonate ion is in position. Slater (1949), however, on the basis of evidence that the —SH inhibitors do not act on the dehydrogenase itself, considers that the shielding phenomenon may need re-interpretation.

Malonate is not a specific inhibitor of S.D. as commonly stated. Thus Quastel and Wooldridge (1928), Cook (1930), and Das (1937) all report inhibition of lactic dehydrogenase by malonate. Stare and Baumann (1939) found that malonate at 0.001M caused (in muscle) a greater inhibition of citrate than of succinic oxidation. Das (1937) showed a slight effect of malonate on

the dehydrogenation of malic acid. Pardee and Potter (1949) also found that malonate depresses oxygen uptake by homogenates in the presence of malic acid, but argue that this is because it blocks the removal of the keto acid product. They believe that malonate inhibition of oxidation in the organic acid cycle is due not only to the succinic block but also to the inhibition of oxidation of oxaloacetate in the presence of pyruvate. On the other hand, there is the evidence for the oxidative formation of succinic acid in muscle brei (Krebs and Eggleston 1940) and plant tissues (Laties 1949*b*) when malonate is present. When such succinic accumulation occurs the block at oxaloacetate must be only of minor importance.

Finally, both Evans, Vennesland, and Slotin (1943) and Liebecq and Peters (1949) agree that malonate inhibits the enzymic decarboxylation of oxaloacetate to pyruvic acid, although the latter authors think that this may be of not much importance in some tissues, in which spontaneous decarboxylation is active. It seems therefore quite likely, at least in experiments with the higher concentrations of malonate, and especially where succinate accumulation cannot be demonstrated, that effects on respiration cannot safely be ascribed simply to the effect on succinic dehydrogenase. The other enzymes shown to be affected by malonate are all concerned with the Krebs cycle. Pardee and Potter (1949), however, ascribe the action of higher concentrations of malonate on oxaloacetate oxidation to the formation of a malonate complex with magnesium. As this is a component of other enzyme systems (e.g. in glycolysis) a malonate effect on such systems cannot be ruled out.

(*b*) *Malonate and the Organic Acid Cycle*

When Gözsy and Szent-Györgyi (1934) published the dicarboxylic acid-hydrogen transport theory for muscle tissue, they pointed out the value of malonate as a specific inhibitor and it has subsequently proved to be a most useful reagent in the study of the organic acid cycle. Thus Krebs (1943) states that the most important experimental observation in support of the existence of this cycle is that succinate can be formed oxidatively from fumarate or oxaloacetate when its own oxidation and its reductive formation from these acids is blocked by malonate. This observation has often been made and from it Krebs argues that during respiration there is a cycle of oxidation in which the dicarboxylic acids arise periodically (see also Green, Loomis, and Auerbach 1948).

Malonate at pH 7.4 does inhibit the oxygen uptake by minced animal tissue and simultaneously succinate accumulates (e.g. Krebs and Eggleston 1940). The picture is not quite so clear, however, when we consider work with intact tissues rather than with cell-free extracts or minced tissue. In 1936 Greville observed that 0.01M malonate at pH 7 was strongly inhibitory to the respiration of minced pigeon breast muscle. But as the addition of physiological concentrations of calcium proved equally inhibitory, he concluded that the tissue had been rendered "unphysiological" by mincing. Accordingly, he repeated his experiments, this time with thin rat diaphragm, damaged very little by

handling. Calcium did not affect the rate of respiration and malonate (0.01M) was only slightly effective. Even at a concentration of 0.05M, inhibition by malonate was incomplete and reached its steady value only after 100 minutes. If the diaphragm were severely damaged by being cut into small pieces, the low malonate concentration (0.01M) produced an immediate response and calcium was likewise inhibitory.

This increased sensitivity of damaged tissue for malonate was also observed by Boyland and Boyland (1936) when investigating tumour respiration. The responses to succinate and fumarate were similarly enhanced by wounding. Weil Malherbe (1937) also found that "the succinic dehydrogenase system seems to be protected from malonate in the structurally intact tissue." Malonate at 0.04M and pH 7 (substrate ketoglutarate) reduced the oxygen consumption of tissue slices by only 50 per cent.; the R.Q. was not affected and succinate did not accumulate. Minced tissue more closely resembled the pure enzyme system in which much weaker malonate, also at pH 7, completely stopped the enzyme action and brought about accumulation of succinate. He considered that "in tissue the effects are complex and cannot all be attributed to a specific action of malonate." However, as we shall see, the anomalous results with tissue are probably due to the use of too high a pH in the medium. The pH optimum for the enzyme reaction is usually quoted as being between 7 and 8; biochemical work on the system is always carried out over this range and experiments with animal tissue generally require alkaline or neutral pH.

Succinic dehydrogenase is an insoluble enzyme and evidence is accumulating to show that it is attached to particles of mitochondrial size (e.g. Chantrenne 1943). It is comparatively easy to obtain a stable suspension from animal cells. In plants, succinic acid is commonly present in small amounts and the acid is certainly metabolized (e.g. Turner and Hanly 1949); there are strong *a priori* reasons for supposing that the enzyme is widely distributed in plant issues. Okunuki (1939) reported its presence in pollen, and Goddard (1944) showed that wheat embryos and their dispersions had a low S.D. activity. Berger and Avery (1943, 1944), however, could not demonstrate its presence in oat coleoptiles, in which other dehydrogenases were active. Bonner and Wildman (1946) showed that the fraction of spinach respiration that is malonate-sensitive is rapidly lost when the tissue is frozen and then thawed and they take this to be evidence for the existence of the enzyme, but in a labile state. This lability, also noted by Goddard (1944), may account for the failure to discover the enzyme in some plant tissues.

In plants, therefore, malonate has been mainly applied to whole plant tissue and in the early work it was usually reported to be ineffective, or even to stimulate respiration. Burris and Wilson (1939) studied the oxygen uptake by *Rhizobium* in phosphate buffer, plus substrate, at pH 6.5. Under such conditions the greater part of the respiration was cyanide-sensitive and cytochrome activity was shown, spectroscopically. It was therefore expected that malonate would prove an effective inhibitor; however, at 0.04M it caused "slow growers"

to increase their rate of oxygen uptake, while with "fast growers" the response to malonate varied from 82 per cent. stimulation to 15 per cent. depression of the oxygen uptake. Results were different in Thunberg experiments. Decolorization of methylene blue, with either glucose or succinate as substrate, was inhibited 40-50 per cent. by 0.04M malonate and inactivation was still evident at 0.01M. The authors took the view that stimulation of respiration might have been due to the malonate combining with copper ions, which inhibit succinic dehydrogenase. They also suggested, on very scanty evidence, that malonate at some concentrations was a good substrate for *Rhizobium*. Again, in 1943, Albaum and Eichel showed that malonate at pH 6 actually stimulated the respiration and also the growth rate of oat coleoptiles by as much as 27 per cent. Malonate thus resembled other organic acids, such as malic, pyruvic, and succinic acids, which are regarded as substrates rather than as inhibitors of respiration. Henderson and Stauffer (1944) also obtained little, if any, inhibition of tomato root respiration by malonate at pH 5.2-5.8 and occasionally they obtained stimulation.

Machlis (1944), using barley root tissue, was the first to obtain consistent inhibition of plant respiration by malonate; he was also the first in this field to use a medium of low pH. It has since been shown that as long as the malonate is applied at low pH (*c.* 4-4.5) is will rapidly inhibit a large part of the respiration in several different tissues, viz. spinach leaf (Bonner and Wildman 1946), carrot root (Turner and Hanly 1947), oat coleoptile (Bonner 1948), barley root (Machlis 1944; Laties 1949*a*), and rhubarb (Morrison 1950). At pH higher than 4.5 the inhibition is less marked and temporary, while at neutral pH there may be stimulation. It is part of the purpose of the present paper to discuss this pH effect but before doing so we shall conclude this review by referring to two other features of importance.

If malonate inhibits respiration through its competitive action on succinic dehydrogenase it should be possible to obtain reversal of the inhibition by the addition of succinic acid or of other acids of the Krebs cycle. Machlis (1944) could obtain no such reversal, although Laties (1949*a*), working also with barley root tissue, did so. Bonner and Wildman (1946) and Bonner (1948) agree with Laties in obtaining reversal (by succinate), but only under certain conditions. Laties (1949*b*) and also Bonner (1948) have provided clear evidence that succinate accumulates in tissues treated with malonate, and this is regarded as strong evidence that an organic acid cycle exists in some plant tissues. Their work will be discussed at greater length after the presentation of our own results.

In all the work so far dealt with, the assumption has been made, and sometimes supported by experimental evidence, that succinic dehydrogenase is coupled with cytochrome oxidase. Bonner (1948), however, has now obtained malonate inhibition in spinach leaf tissue, for which he presents reasons for believing that the terminal oxidase is polyphenol oxidase. Rosenberg and Ducet (1949) and Stenlid (1949) have thrown doubt on this latter conclusion and further work along these lines is obviously desirable.

II. Cytochrome Oxidase and Succinic Dehydrogenase in Carrot Tissue

Both these enzymes have been prepared from carrot root phloem parenchyma. Details will not be presented here† but the results merit brief report. A crude preparation was obtained by maceration of the tissue in a Waring Blendor in M/15 phosphate buffer, pH 7.4, followed by centrifugation of a muslin filtrate of the blend; it oxidized cytochrome *c* in the presence of *p*-phenylenediamine, the optimum pH being between 7.2-7.8; the Michaelis constant varied from 3.5 to 4.8×10^{-6}M, of the same order as that calculated by Goddard (1944) for the cytochrome oxidase of wheat embryo. The enzyme was inhibited by KCN (10^{-3}M) and there was light-reversible inhibition by carbon monoxide. The same extract would not oxidize succinic acid but an active succinic dehydrogenase was prepared by similar methods when greater care was taken to conduct all preparatory operations at 5°C. This extract brought about oxidation of succinic acid in the presence of either methylene blue or cytochrome *c*. The optimum pH for the reaction was 7.3. The enzyme activity between pH 6.2 and 8.3 was partially inhibited by 0.01M malonate and this inhibition was shown to be competitive. These facts therefore provide justification in what follows for assuming that malonate inhibition of respiration is, in part at least, due to the inhibition of succinic dehydrogenase; also that the succinate-dehydrogenase of the carrot root is inhibited *in vitro* by malonate at high pH.

III. Effect of Malonate on the Respiration of Carrot Root Tissue

(*a*) *Experimental Methods and Material*

The plant material was secondary phloem parenchyma from the storage roots of carrot (Danvers Half-long variety). For measurement of respiration, discs 1 mm. thick, 0.8 cm. diameter, were cut by microtome and cork borer and aerated in distilled water for periods up to 400 hours. Oxygen uptake and carbon dioxide output were measured by the two-vessel method of Warburg, values for the R.Q. in media of pH greater than 6 being checked by acid tip. The methods were exactly as reported in a previous paper (Turner and Hanly 1949). As solutions of buffer salts may themselves have marked effects on the respiration rate, the tissue was usually suspended in distilled water and the malonate solution added later from the side-arm. The pH of the medium was measured at the start and finish of each experiment. For some of the work at low pH the tissue was maintained throughout in M/15 phosphate buffer.

Previously a distinction has been made between two types of carrots used in these experiments; type *A*, the respiratory rate of which falls gradually after 80-100 hours of aging in water, to a value below $Q_{O_2}^{FW*} = 100$; and type *B* with a much higher respiration rate remaining above $Q_{O_2}^{FW} = 160$. It is believed that

† This work will be separately reported by K. S. Rowan.

* $Q_{O_2}^{FW}$ = cu. mm. of oxygen consumed per g. fresh weight of tissue per hour.

the differences between carrot root tissue of types *A* and *B* are quantitative rather than qualitative and results with both types are treated together.

In preliminary experiments it was found that the effects of malonate may vary with the age (hours from cutting) of the tissue slice. Hence, usually one large batch of discs were cut and washed continually in aerated distilled water and each experiment (three or four treatments and controls) was repeated at intervals of several days with slices taken from this batch.

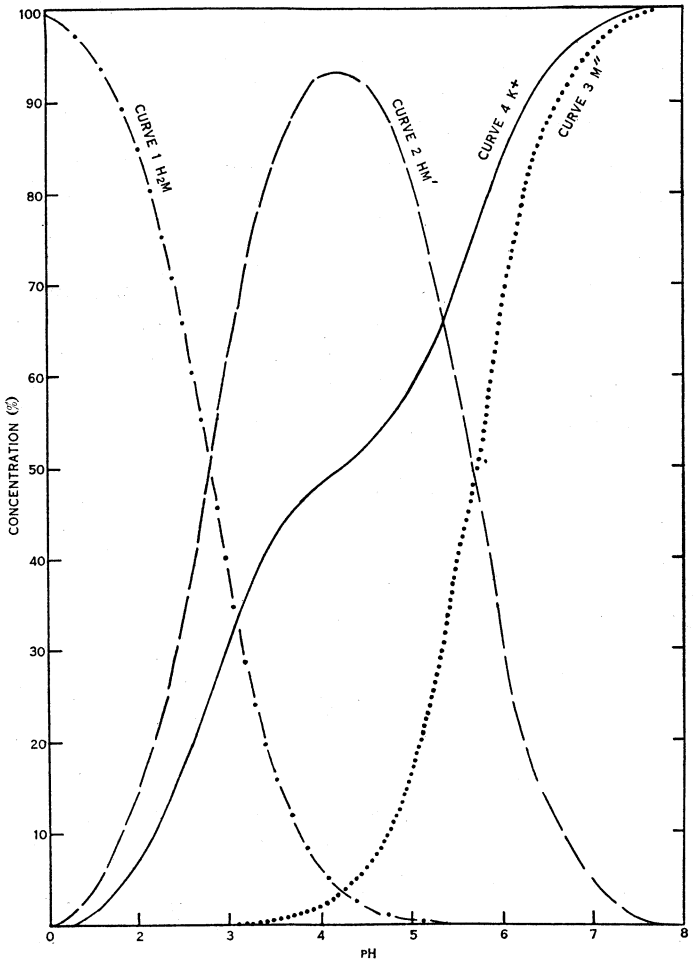

Fig. 1.—Dissociation curves for potassium malonate.
1. Percentage concentration of undissociated malonic acid.
2. Percentage concentration of monobasic malonate ion.
3. Percentage concentration of dibasic malonate ion.
4. Half percentage concentration of potassium ion (thus at pH 4, $K^+ = 96.2$ per cent. of the total molarity of the solution).

Like succinic acid, malonic acid is a weak dibasic acid and in view of the results already obtained for succinate (Turner and Hanly 1949) it was con-

sidered important to test the effect of malonate over a range of pH. The relative concentrations of undissociated malonic acid (pK_1 2.5; pK_2 5.7) and of its ions at increasing pH have been calculated and are presented in Figure 1. We also include the curve for potassium ion concentration over the same range, since the pH was adjusted by adding potassium hydroxide to the acid solution. These curves have been used to determine the molecular and ionic concentrations to be quoted.

(*b*) *Respiratory Effects of Malonate applied at pH* 4-4.5

(i) *Oxygen Uptake (pH* 4-4.5)

As already reported (Turner and Hanly 1947) we have found that malonate causes a clear-cut and continuous depression of carrot root respiration only when it is applied at or near pH 4. The effect is illustrated in Figures 2, 4, 6, 7, 8, 9, 11, 12, and 13, and in Table 3.

In another paper (Turner and Hanly 1949) it has been shown that phosphate buffers at pH 4.5 neither stimulate nor depress the rate of the oxygen uptake in carrot root tissue; succinate at pH 4 brings about maximal stimulation and neutral phosphate buffers may also stimulate carrot root respiration. In our experiments we have only obtained *inhibition* of respiration at *low* pH when inhibitors such as malonate or cyanide are also present in the external solution and we shall therefore take it that these inhibitions are not due to the low pH *per se,* but to the presence of the inhibitor. The effect of malonate at pH 4.5 is the same whether the acid is supplied in water or in M/15 KH_2PO_4 phosphate buffer.

There is some variation in the sensitivity to malonate for slices of different age and for tissue from different batches. As a result of numerous experiments over three years, we may generalize as follows. Malonate at pH 4-4.5, from 0.005M to 0.01M, causes only slight depression of the oxygen uptake; minimal values are reached within 20-30 minutes, after which recovery takes place to the normal $Q_{O_2}^{FW}$ or above it. Malonate at 0.03-0.05M depresses the $Q_{O_2}^{FW}$ to a value that is reached about 30 minutes after application of the inhibitor and is maintained steady for at least four hours subsequently. The inhibition curves for 0.04M and 0.05M malonate are substantially similar to that for 0.03M, although the steady values may be slightly lower. The mean steady value given by 0.05M malonate (which we regard as a critical concentration) in 29 experiments with widely different batches of carrot tissue was $Q_{O_2}^{FW} = 37.3$, S.E. 2.02 (total $Q_{O_2}^{FW}$ of controls 72-230).

When the malonate concentration is increased from 0.05M towards 0.1M the $Q_{O_2}^{FW}$ is suppressed significantly below this value, and moreover, the inhibition time curve now shows a continuous downward trend. Such solutions obviously damage the cells irreversibly; they lose turgor and some of their contents, the solution bathing them becoming yellowish and opalescent. The effects summarized above are illustrated in the results for a single experiment in which

nine comparable sets of slices were subjected to different malonate concentrations, the pH being maintained at 4.5 by phosphate buffer (Fig. 2).

These results are similar to those obtained by other workers with different tissues. The concentration of malonate required to produce marked inhibition of oxygen uptake is near 0.05M for carrot root, barley root (Machlis 1944; Laties 1949*a*), spinach leaf (Bonner and Wildman 1946), and for starved *Avena* coleoptiles (Bonner 1948). Normal *Avena* coleoptiles require near 0.2M malonate for 90 per cent. inhibition. In all these experiments inhibition was greatest at pH 4-4.5. So far, only in *Arum* spadix tissue has respiration been shown to be unaffected by malonate at low pH (James and Beevers 1950).

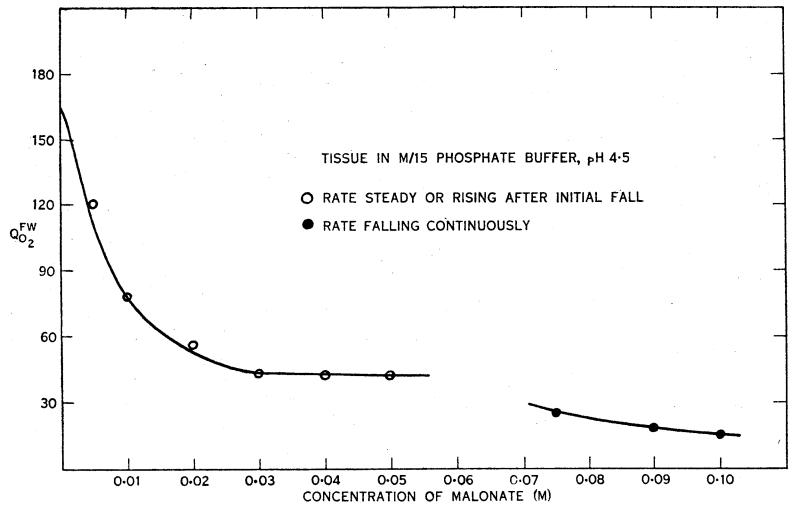

Fig. 2.—Relation between respiration rate (two hours after addition of malonate) and malonate concentration, pH 4.5. All slices of same age and in M/15 phosphate buffer. Initial rate in buffer (mean), $Q_{O_2}^{FW} = 165$.

In none of the papers quoted is it stated that inhibition of oxygen uptake is complete, even with high concentrations of malonate. In our own experiments the results strongly suggest that there is a basal malonate-resistant respiration system and that variations in total Q_{O_2} are mainly due to variations in the malonate-sensitive system. If this is so, then as Commoner (1940) has pointed out, comparisons of percentage inhibition have little meaning. In earlier work (Robertson and Turner 1945) we have postulated the existence of a cyanide-resistant basal respiration and more recently, using carrot roots of the same stock as those treated with malonate, we have found that cyanide at 5×10^{-4}M brings down the $Q_{O_2}^{FW}$ to a basal rate that is not decreased when the cyanide concentration is increased to 10^{-3}M. The effect of cyanide therefore resembles that due to malonate, with one possibly important difference. The basal respiration in malonate remains steady with time, whereas that in cyanide shows usually a very slow but significant, almost linear, fall with time.

In his discussion, Commoner (1940) has adopted the hypothesis that for any tissue the cyanide-stable respiration (b) remains at a steady value while the cyanide-sensitive respiration (x) varies widely. He therefore plots y (total R) against x, obtaining a straight line, at 45° slope, cutting the ordinate at the value of b.

In our experiments with both cyanide and malonate there was a suggestion that the level of the basal respiration varied slightly with the age of the slices and with the rate of total respiration. The data for the group of experiments in which both inhibitors were used have therefore been analysed. We use the following symbols:

TR = total respiration ($Q_{O_2}^{FW}$),
BR_c = basal rate (extrapolated to zero time) in cyanide ($Q_{O_2}^{FW}$),
BR_m = basal rate (mean) in malonate ($Q_{O_2}^{FW}$),
A = age of tissue in hours from cutting.

For 29 experiments with malonate the simple correlation coefficients were: BR_m/TR, $r_1 = 0.427$; BR_m/A, $r_2 = 0.169$; TR/A, $r_3 = 0.152$. Of these, only r_1 is significant and at the 2 per cent. level. Calculation of partial correlation coefficients shows also that there is no significant linear correlation between BR_m and A (eliminating TR) and that for BR_m and TR (eliminating A) the coefficient is 0.4123, significant at the 5 per cent. level. Thus the effect of equalizing for age is to reduce the significance of the correlation between BR_m and TR to the 5 per cent. level, from the 2 per cent. level. Experiments with carrot tissue over several years have shown that there is a relationship between age of slice and TR, but it is complex, as both young and old slices may have a lower rate of respiration than slices of intermediate age. For the experiments under review, however, all that is established is that there is a linear relationship between BR_m and TR, the regression equation being

$$BR_m = 0.109 \times TR + 21.44.$$

Thus an increase in total respiration is accompanied by a small but significant increase in the rate of the basal respiration.

For comparison of the effects of malonate and cyanide we have plotted the regression of BR_m and of BR_c on TR (Fig. 3). The regression coefficients were 0.109 and 0.152 respectively and these are significant, but not significantly different from each other. Therefore, a common regression coefficient was calculated for the 29 malonate and the 53 cyanide experiments, the regression lines being:

$$BR_m = 37.3 + 0.131\ (TR - \overline{TR});\ \overline{TR} = 146.0$$
$$BR_c = 49.7 + 0.131\ (TR - \overline{TR});\ \overline{TR} = 126.3.$$

Thus both for cyanide and malonate the basal respiration shows the same increase with increased TR. The means for BR_m and BR_c (37.3 and 49.7) are significantly different and this suggests that the fraction of the respiration insensitive to cyanide is quantitatively different from that insensitive to malonate. This by no means follows, however. There is considerable difficulty in decid-

ing what figure to use for BR_c as the rate in cyanide continually declines,* whereas that in malonate is steady after two hours. Hence our figures for BR_c, obtained by extrapolation to zero time in cyanide, are not strictly com-

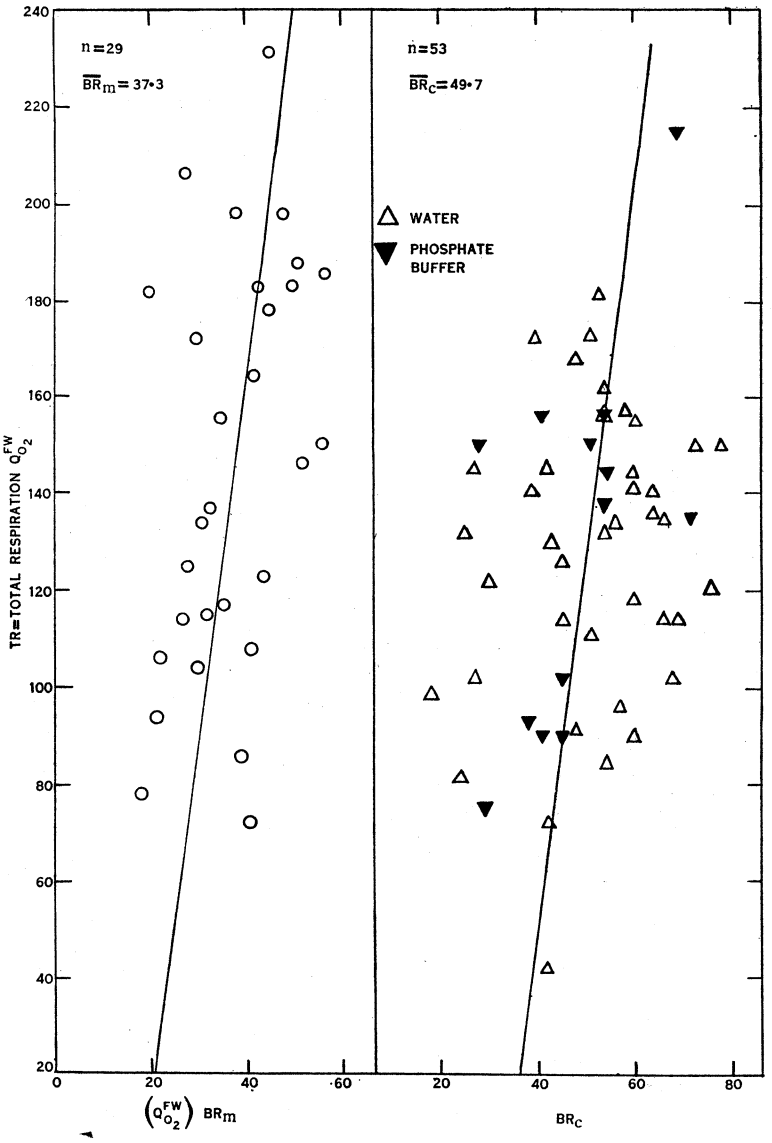

Fig. 3.—Regression of basal respiration in malonate (BR_m) and in cyanide (BR_c) on total respiration.

parable with those for malonate. By arbitrarily choosing a time at which to determine BR_c (say four hours after addition of cyanide) it would undoubtedly

* This feature is not brought out in Figure 4, for which the basal respiration in cyanide is atypically steady.

be possible to obtain equality of $\overline{BR}_c$ and $\overline{BR}_m$; for obvious reasons, this has not been done. It must also be borne in mind that, as both inhibitors form dissociable complexes with their respective enzymes, complete inhibition of the sensitive fractions of the respiration will not be obtained, inhibition being "only as complete as is required by the dissociation law" (Warburg 1949).

(ii) *Respiratory Quotients in Malonate* (0.05*M*; *pH* 4)

Respiratory quotients were measured in some experiments by the two-vessel method of Warburg. Table 1 gives figures and Figure 4 presents the graph for a typical experiment. An initial gush of CO_2 was always obtained on tipping malonate solutions at pH 4 into distilled water surrounding the tissue slices. We have previously (Turner and Hanly 1949) explained this as due to the increased acidity of the tissue medium expelling CO_2 from bicarbonate present in the medium and tissue. This initial gush (first 20 mm. reading) was not included in calculations of the mean R.Q. The data are consistent; while the malonate at pH 4 rapidly reduces the rate of oxygen uptake, the rate of CO_2 output declines more slowly and the R.Q. rises rapidly to about 3.

TABLE 1

$Q_{O_2}^{FW}$ AND R.Q. OF CARROT ROOT TISSUE IN CONTACT WITH 0.05M MALONATE AT pH 4

Carrots	Time from Cutting (hr.)	pH of Malonate	$Q_{O_2}^{FW}$ in Dist. Water Prior to Malonate	$Q_{O_2}^{FW}$ in Malonate	R.Q. Range in Malonate	Mean R.Q. in Malonate
Type *A*	1	4.2	90	30	2.8-3.6	3.0
	20	4.0	132	36	2.9-3.5	3.2
	20	4.2	183	60	2.6-2.9	2.7
	20	4.2	183	48	—	2.7
	20	4.1	204	45	2.6-3.6	3.2
Type *B*	48	4.3	198	42	3.0-3.8	3.3
	70	4.2	—	36	1.6-3.6	2.6

Figure 4 illustrates the results of an experiment in which the respiratory quotients of tissue in malonate and in cyanide were measured concurrently. The cyanide solution had a pH of about 5.5 and did not produce the initial gush of CO_2 always observed with the more acid malonate solutions. The mean quotients for both inhibited tissues were high (*c.* 2.5) and not significantly different. Cyanide inhibition is known to be accompanied by aerobic fermentation in some plant tissues and we presume the same to occur with malonate. The presence of alcohol has been detected in malonate-poisoned tissue, but so far no quantitative estimates have been made.

These results may be compared with those of other workers. Machlis (1944) publishes one curve showing marked inhibition of both oxygen uptake and CO_2 output by malonate in barley roots, but the final R.Q. in malonate (0.05M) is nevertheless 1.6 as against 1.0 for the control and 2 for cyanide (0.005M). Malonate at 0.01M gave about 50 per cent. inhibition and an R.Q. of 1.14. On the other hand, Bonner and Wildman (1946) (one experiment

quoted) found that malonate inhibited the oxygen uptake of spinach leaf by 90 per cent. and CO_2 evolution by approximately the same amount. R.Q. data are not available for *Avena*. Laties (1949*a*) stated that in his work with barley roots the R.Q. in malonate was very close to the control. However, the only figures quoted show a 24 per cent. decrease of Q_{O_2} and a 5 per cent. decrease of Q_{CO_2}, the R.Q. being markedly changed from 0.82 to 1.03 (with fumarate and malonate added the R.Q. returned to 0.79).

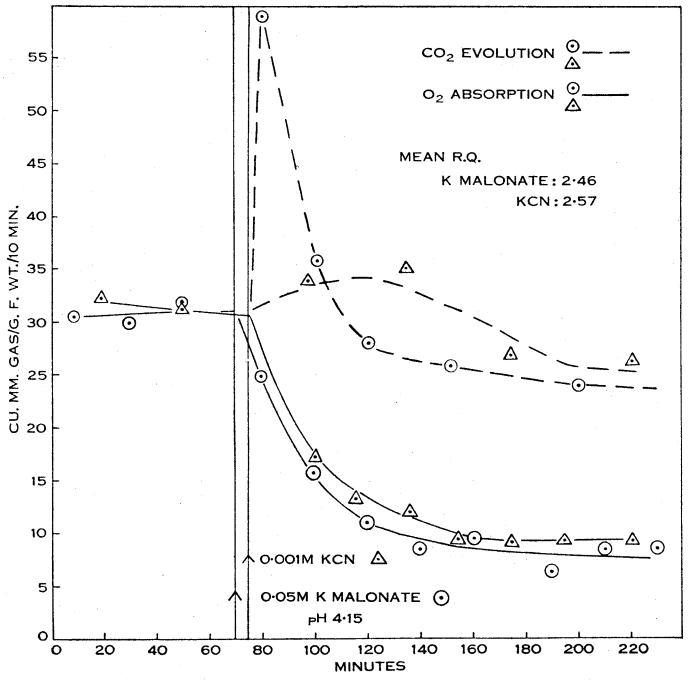

Fig. 4.—Comparison of rates of gas exchange in 0.05M malonate, pH 4.15 and in 0.001M KCN. Mean R.Q. calculated with omission of first two readings. Tissue used 44 hours after cutting.

(iii) *Inhibition of Salt Respiration (pH 4-4.5)*

It is known that when carrot root respiration is stimulated by neutral salts (e.g. KCl), the respiration is depressed to the "basal" level by cyanide (Robertson and Turner 1945) and by carbon monoxide (Weeks and Robertson 1950). Malonate at 0.05M and pH 4 has the same effect on the salt-stimulated respiration as have the other two inhibitors. The level of the basal respiration is not altered in the presence of KCl although, of course, the percentage inhibition of the total respiration is markedly changed.

(iv) *Reversibility of the Malonate Inhibition (pH 4-4.5)*

Self reversal.—When carrot root slices are supplied with malonate at pH 4 at a concentration of 0.005-0.01M, the inhibition is not complete and recovery towards (or even above) the normal rate takes place in the presence of the inhibitor (Fig. 5).

Slow recovery in weak solutions of malonate is suggested by Figure 6 of Laties's (1949*a*) work on barley root; it is also shown in our own experiments when stronger malonate solutions are applied at pH 5 or 6 (see below).

On removal of malonate in the external solution.—The inhibitory effect of malonate on succinic dehydrogenase *in vitro* is reversible if the enzyme is washed with water (Hopkins, Morgan, and Lutwak Mann 1938). The inhibition of the respiration in carrot tissue is also removed if the applied malonate solution is replaced with water.

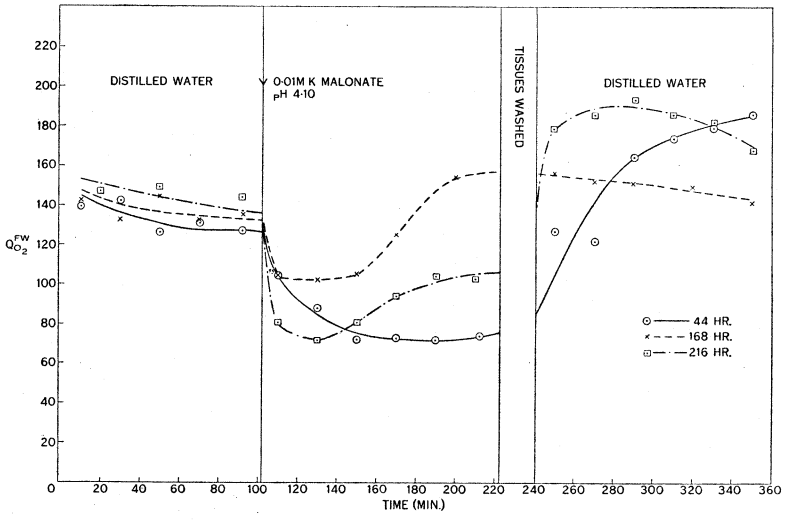

Fig. 5.—Self-recovery from partial inhibition of respiration by 0.01M malonate, pH 4.1, and the effects of replacing this by water. Tissue used 44, 168, 216 hours after cutting.

Reversibility was first tested in those experiments in which malonate at concentrations ranging from 0.05M to 0.03M brought the respiration down to the steady basal rate. Each set of slices was treated with the malonate solution for two hours. The tissues were then quickly rinsed with four successive changes of distilled water and the drifts of the rates of oxygen uptake were subsequently followed in distilled water. Results are graphed in Figures 6 and 7. It is clear that recovery takes place; the rate of recovery is a function of the age of the tissue slice (Fig. 6) and it is shown that, except with aged discs (with 0.05M malonate), the final rate attained is greater than that before inhibition.

During the inhibition of respiration the R.Q. is high (*c.* 3) but after washing it falls rapidly. It is, however, important to note that even after the respiration has risen to or above its normal value, the R.Q. remains near 1.3.

The self recovery noted above is accelerated and its extent usually increased if the weak malonate solution is replaced by water (Fig. 5).

Reversal by addition of succinic acid; competitive inhibition.—If malonate does not affect glycolysis and acts by inhibiting one step only of the Krebs cycle, and if the inhibition is competitive, one might expect to obtain reversal of inhibition by adding succinic acid or indeed other acids that give rise to this in the cycle, e.g. fumaric acid. In several early experiments (Turner and Hanly 1947), mostly with 0.05M malonate, we were not able to obtain such reversal or any indication that the inhibition was competitive. Thus the rate of oxygen uptake of malonate-poisoned tissue was not affected by the presence of succinic or fumaric acids, even when these were present in concentrations up to five times that of the malonate. This obtained whether these acids were added to the tissue before, with, or after the malonate. These negative results of reversal experiments were in accordance with those of Machlis (1944) for barley roots, but are in disagreement with the more recent findings of Bonner and Wildman (1946), Bonner (1948), and Laties (1949*a*). Late publication of the present paper has allowed a fuller investigation of this aspect of the problem.

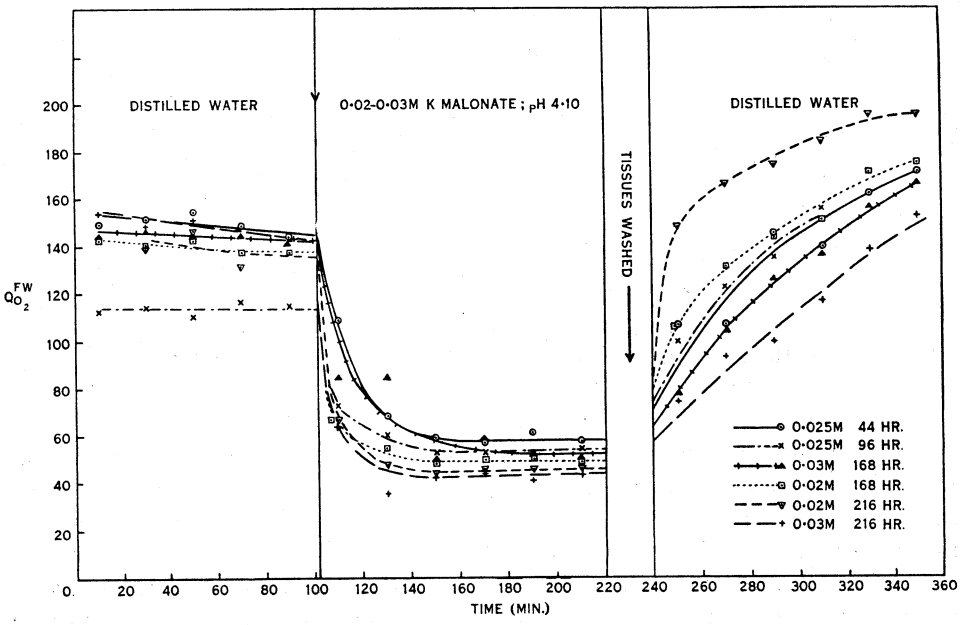

Fig. 6.—Inhibition of respiration by 0.05M malonate, pH 4.1, and recovery following replacement of malonate by distilled water. Tissue used 44-216 hours after cutting.

These new experiments were done with roots from a batch of Danvers Half-long carrots dug in November and also with carrots bought on the open market. Similar results were obtained from all samples. The technique was as described already except that respiration was measured in M/15 KH_2PO_4 buffer at pH 4.5, with or without inhibitor and succinic acid. In all experiments the rate of oxygen uptake in buffer alone was measured before the acids were

tipped from side-arms; in experiments of type *A* (Fig. 10) the malonate was added first, the succinate (or water as control) later. In type *B* (Fig. 10)

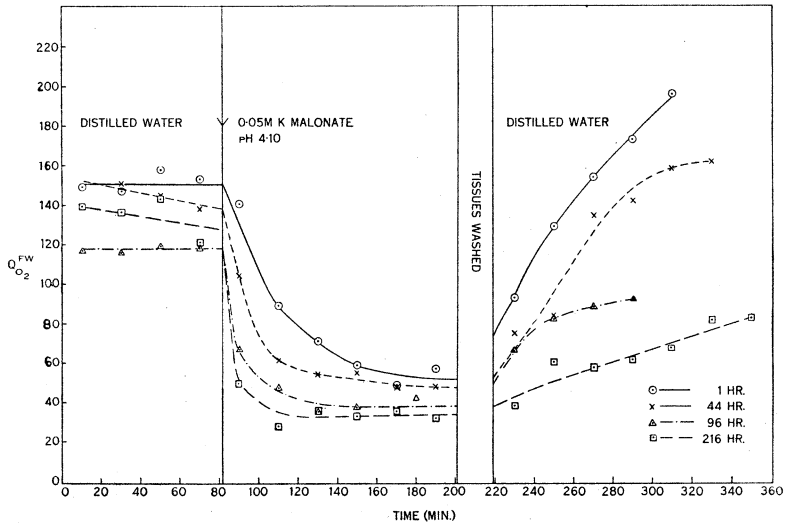

Fig. 7.—Inhibition of respiration by 0.02-0.03M malonate, pH 4.1, and recovery following replacement of malonate by distilled water. Tissue used 1-216 hours after cutting.

both acids were added together, and controls with single acids were run at the same time.

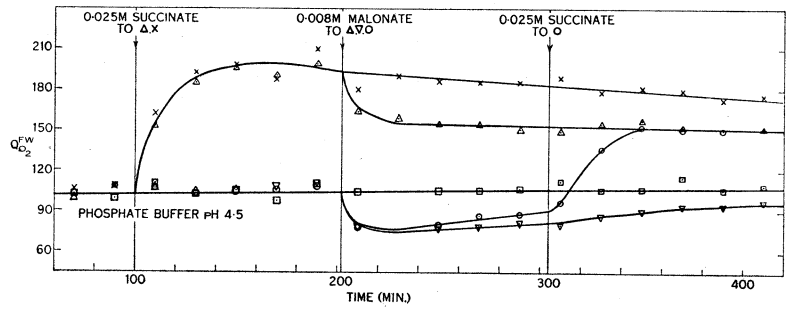

Fig. 8.—Experiment illustrating reversal of malonate inhibition of respiration by succinate and competitive inhibition. Five sets of carrot root slices, 310 hours washed, in M/15 phosphate buffer pH 4.5. Malonate at 0.008M, which does not bring the $Q_{O_2}^{FW}$ to the basal level even in the absence of succinate.

Examples of the results are plotted in Figures 8, 9, and 10, and a concise summary of all these experiments is given in Table 2 and Figure 10. The single figures for Q_{O_2} in this table represent the mean values during each period of treatment, excluding values for transition periods (e.g. the first 30 minutes after tipping acids). Most of these figures are means of 12 ten-minute readings.

TABLE 2

REVERSAL OR PREVENTION OF MALONATE INHIBITION BY SUCCINATE. ALL EXPERIMENTS WITH M/15 KH_2PO_4, pH 4.5

Type *A* experiments, malonate added first, succinate later (reversal)

			Malonate			Succinate plus Malonate			Succinate	
Expt.	Hours from Cutting	Initial Rate $Q^{FW}_{O_2}$	Malonate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	Succinate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	$Q^{FW}_{O_2}$	Percentage of Initial Rate
W 46/2	290	108	0.008	82	76	0.025	138	128		
3	310	106	0.008	85	80	0.025	150	142		
4	335	116	0.008	99	85	0.025	154	133		
W 52/1	93	160	0.05	37	23 } Mean	0.025	36	23 } Mean		
		173		39	23 } **21**	0.01	34	20 } **21**		
		160		28	18 }	0.008	30	19 }		
		136	0.01	84	62	0.025	178	131		
		152		96	63	0.01	206	136		
		143		101	71	0.008	211	148		
		160	0.008	80	50	0.025	201	126		
		155		78	50	0.01	171	111		
		178		104	75	0.008	191	107		
		147	—			0.025			244	166
W 52/2	117	135	0.03	33	24 } Mean	0.025	41	30 } Mean		
		148		36	24 } **26**	0.008	44	30 } **30**		
		159		44	28 }	0.001	46	29 }		
		145	0.01	88	61	0.025	220	152		
		127		71	56	0.008	174	137		
		140		87	62	0.001	174	124		
		149	0.005	109	73	0.025	210	141		
		141		94	67	0.008	202	143		
		155		111	72	0.001	192	124		
		148	—			0.025			230	156
W 79/1	140	177	0.05	112	63	0.025	188	106		
		173		110	64		185	107		

TABLE 2 (*Continued*)

Type *B* experiments, malonate and succinate added simultaneously (competition)

Expt.	Hours from Cutting	Initial Rate $Q^{FW}_{O_2}$	Malonate: Malonate Concentration (M)	Malonate: $Q^{FW}_{O_2}$	Malonate: Percentage of Initial Rate	Succinate plus Malonate: Succinate Concentration (M)	Succinate plus Malonate: $Q^{FW}_{O_2}$	Succinate plus Malonate: Percentage of Initial Rate	Succinate: $Q^{FW}_{O_2}$	Succinate: Percentage of Initial Rate
W 46/2	290	99	0.008			0.025	160	162		
		110	0.008	82	76	—				
W 52/3	235	143	0.01			0.025	174	122		
		137	0.01	126	92	—				
W 53/1	65	144	0.005	106	74	—				
		167	0.01	93	56	—				
		170	0.01			0.025			228	137
		172	0.01			0.005			214	124
		167	0.01			0.001			167	100
		162	0.005			0.025	262	162		
		135	0.005			0.005	226	167		
		166	0.005			0.005	138	83		
		174	0.01			0.025	243	140		
		171	0.01			0.005	198	116		
		190	0.01			0.001	134	70		
W 58/1	90	110	0.01	59	54	—				
		109	—			0.025			241	221
		111	0.01			0.025	97	88		

It was not always practicable to run a control set of slices in buffer throughout, so all comparisons are based on initial readings in buffer (see Fig. 8).

These results make it clear that for carrot tissue, inhibition by malonate is reversible so long as concentrations of malonate are used that are not high enough to inhibit completely to the ground level. The inhibition is apparently of the competitive type, its extent being much reduced when malonate and succinate are added together—in fact, a mixture of the two acids usually brings the respiration rate above the initial steady value in buffer. Failure of reversal was obtained in two experiments, in both of which the malonate (0.03-0.05M) brought the respiration to the "ground" level (Figs. 9, 10). Reversal was always obtained when the respiration had been inhibited by malonate to a lesser extent, even for instance in Experiment W 79 (Table 2), when the malonate was at 0.05M and the succinate only 0.025M.

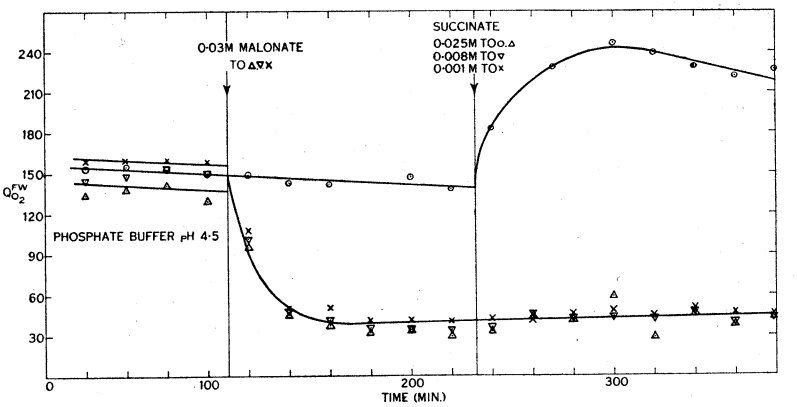

Fig. 9.—Experiment illustrating lack of reversal by succinate of malonate inhibition of respiration, when the malonate concentration (0.03M) has brought the respiration down to the ground level. Four sets of carrot root slices, 117 hours washed in M/15 phosphate buffer at pH 4.5.

In the experiments (type *A*) with weak malonate (0.005-0.01M) self reversal of inhibition had begun before the succinate was added, but nevertheless the marked effect of succinate was not thereby obscured.

Figure 10 shows the relationship between the concentration of succinate and the degree of reversal of malonate inhibition. It is, of course, possible that still higher concentration of succinate might cause reversal of the inhibition brought about by 0.05M malonate. As stated above, such reversal with the concentration ratios of 0.05M malonate/0.25M succinate was not obtained in earlier work. It is, however, difficult to test this point adequately because high acid concentration (near 0.2 or 0.3M) is harmful to the tissue. The simplest explanation of all our results (and of those of Machlis) is that malonate concentrations sufficiently high to bring the respiration down to the ground

level inhibit succinic dehydrogenase and also other enzyme systems concerned in the organic acid cycle; reversal with succinic is therefore not to be expected.

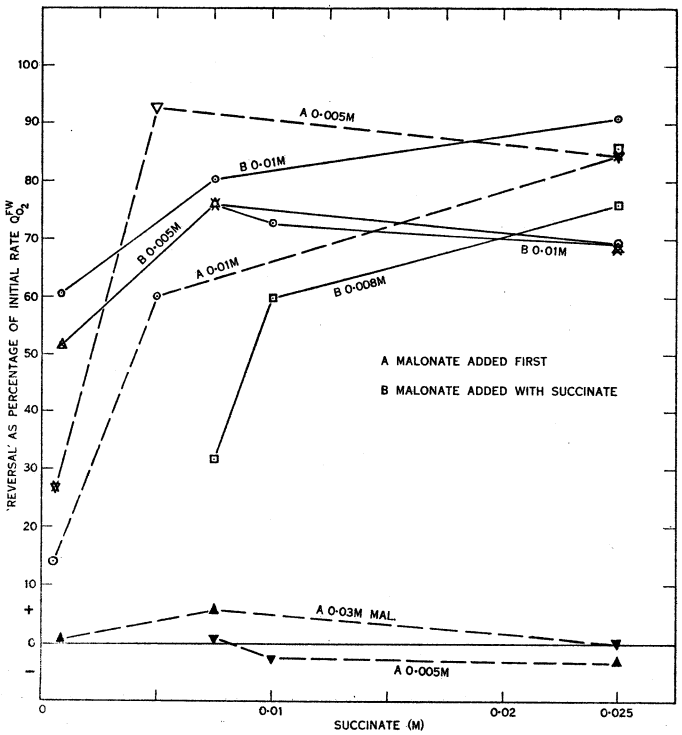

Fig. 10.—Effect of succinate in reversal of malonate inhibition. Succinate concentration plotted against the difference between $Q_{O_2}^{FW}$ malonate and Q_{C}^{FW} malonate + succinate, expressed as a percentage of the initial rate in phosphate buffer. *A*, malonate added first; *B*, malonate and succinate added together. The figures on the lines give the concentration of malonate.

It should be noted that the difference between the later experiments and the earlier was not due simply to the presence of a buffer solution; in the first place we obtain no reversal in buffer solution if the malonate has inhibited to the basal rate; secondly, we have confirmed reversal of malonate inhibition for carrot root slices unbuffered by phosphates, using 0.01M malonate and 0.025M succinate.

(v) *Effects on the Organic Acid Content of the Tissue* (*pH* 4-4.5)

Bonner (1948) and Laties (1949*b*) found that for oat coleoptiles, spinach leaves, and barley roots the normal low level of succinic acid in the tissues is raised following the addition of malonate at pH 4.5. The succinic acid accumulation is regarded as due to its oxidative formation from pyruvate and

4-C dicarboxylic acids, either those present in the cell or those formed by CO_2 fixation with pyruvate. In some spinach samples and in barley roots, added pyruvate and fumarate enhance the succinic accumulation associated with malonate.

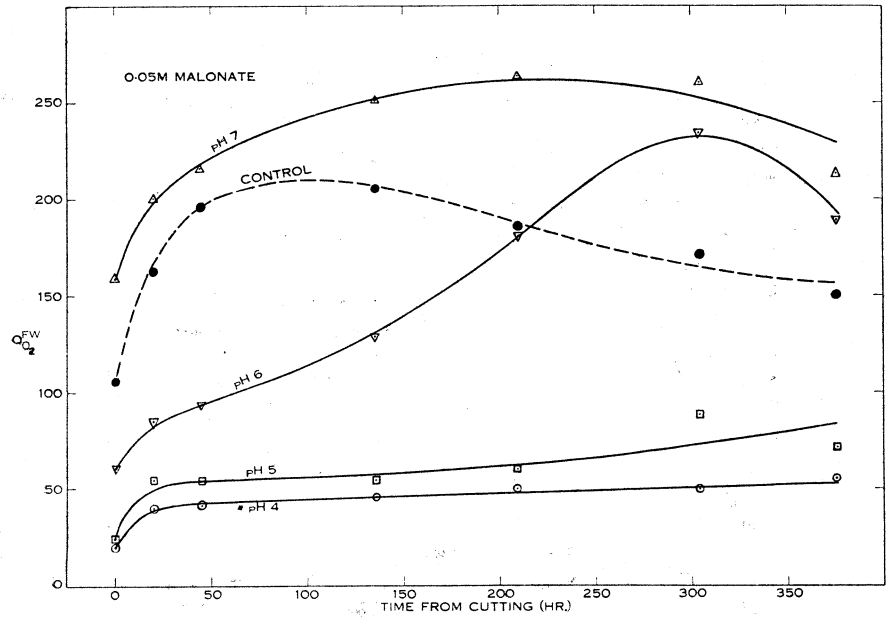

Fig. 11.—Drift of the respiratory response of carrot root tissue towards 0.05M malonate with time from cutting of the slices. The values plotted are the minimum (or maximum) rates of oxygen uptake achieved in the several experiments; control, tissue in distilled water. Each point gives the result of a four-hour experiment with tissue taken from the batch washing in water (see Figs. 12, 13).

Experiments along these lines have been carried out in this laboratory with carrot tissue, the organic acids being detected and, in some experiments, quantitatively estimated by the methods of Lugg and Overell (1948) and Bryant and Overell (1951).* The carrot root tissues, as used in our respiration experiments, invariably contained malic acid (whose concentration fell with age from cutting); in most samples smaller but measurable quantities of succinic acid were also present.

In six experiments we have only twice obtained any accumulation of succinate as a result of the addition of malonate (0.015-0.05M, pH 4.0); in neither case was the increased succinate content significant. In the other four

* The tissues were extracted with 80 per cent. boiling ethanol and the extract, after removal of alcohol, passed through an Amberlite I R A 400 column. Anions were eluted with 1N ammonium carbonate, and acids assayed by paper chromatography. We are indebted to Mr. Overell for advice and assistance in these experiments.

experiments there was no change in succinate concentration, although in two of them the malonate was added to the tissue respiring in an atmosphere of 4 per cent. carbon dioxide, in an attempt to promote carbon dioxide fixation. In all these six experiments the tissues were shown to contain malonic acid after treatment with this acid at pH 4 and the malonate supplied to replicate samples caused inhibition of respiration.

(*c*) *Effects of Malonate at pH* 5-7

(i) *Oxygen Uptake (pH* 5-7)

The results of a series of experiments, in which both the pH of malonate (0.05M) and the age of the slices were varied, are plotted in Figure 11. It will be clear that the effects of solutions more alkaline than pH 4.5 are different from those already described. Numerous experiments confirm those of Table 3 and the effects may be summarized as follows:

TABLE 3

RESPIRATION RATES FOR ONE TIME-SERIES OF EXPERIMENTS WITH 0.05M K MALONATE SOLUTIONS, pH 7-4

	Respiration rates (cu. mm. O_2/g. fresh wt./hr.)				
Time from Cutting (hr.)	Control in Water*	pH 7 Maximum Rate	pH 6 Minimum or Maximum Rates, i.e. Turning Point of Curve	pH 5 Minimum Rate—Turning Point of Curve	pH 4 Minimum Rate
1	106.2	159.0	60.0	24.0	21.0
20	165.0	201.0	84.0	54.0	40.0
44	197.4	216.0	93.0	54.0	42.0
136	206.4	252.0	129.0	54.0	47.0
208	185.0	264.0	180.0	60.0	50.0
304	172.2	261.0	234.0	87.0	50.0
376	142.8	213.0	189.0	72.0	56.0

* Mean of the steady rates of the four tissue sets in distilled water before malonate solutions were tipped.

Malonate 0.05*M, pH* 5 (0.5 *per cent. undissociated molecule* H_2M, 82 *per cent. monobasic ion* HM'.—This solution has an effect similar to that of weaker malonate at pH 4 (0.01-0.02M). It slowly depresses the oxygen uptake (but not to the basal level). There is always, however, a reversal of the inhibition with time, the rate rising during a four-hour period towards the control rate in water (Figs. 12, 13). The minimum rate reached is a function of the age of the tissue slices, tending to rise from $Q_{O_2}^{FW} = 50$ (or in a few experiments, 20) to 80 as the age increases from 1 to 400 hours, Table 3). The rate of recovery from the initial depression is greater for the older slices (Fig. 13). The pH of the external malonate solution shows little change during these experiments.

Laties (1949*a*) showed also that in barley roots the inhibition at pH 5 falls off with time.

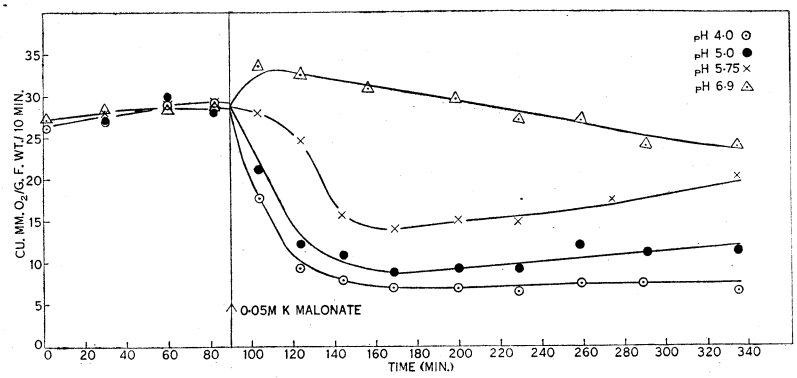

Fig. 12.—Reactions of tissue respiration towards potassium malonate solutions (0.05M) at four different pH. Tissue used 20 hours after cutting.

Malonate 0.05*M*, *pH* 6 (0.02 *per cent. undissociated molecule* H_2M, 32 *per cent. monobasic ion* HM').—This solution has effects that vary markedly with the age of the slices (Figs. 11, 12, 13). Tissue that has been cut less than 200 hours reacts in the same way as tissue supplied with solutions at pH 5,

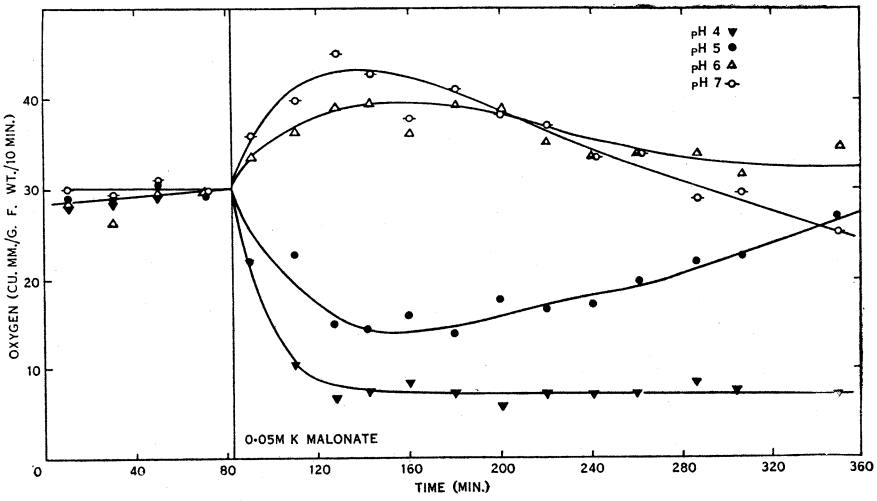

Fig. 13.—Reactions of tissue respiration towards potassium malonate solutions (0.05M) at four different pH. Tissue used 308 hours after cutting.

except that the rate of inhibition is less and the minimum value reached before recovery is higher. At 200 hours the solution at pH 6 has no apparent effect on the oxygen uptake, whereas after this time, stimulation is obtained as with neutral malonate (Figs. 3, 4). These solutions, at pH 6, did not show any marked drift of pH with time during the experiments.

Malonate 0.05*M*, *pH* 7 (*salt completely dissociated*).—This solution consistently causes an initial stimulation of the oxygen uptake (Figs. 11, 12, 13, 14); subsequently the rate returns either to that normal in water or to a value slightly below this. The stimulation is caused whatever the age of the tissue slices, although it is greater for the older tissues. During these experiments the pH of the external solutions falls to about 6.

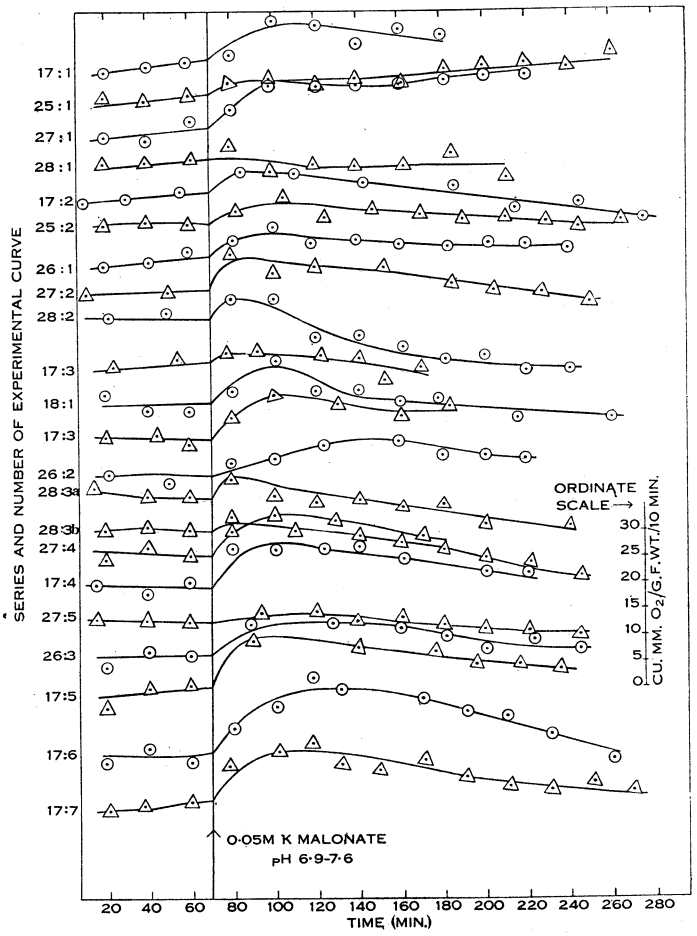

Fig. 14.—Reaction of tissue respiration towards solution of potassium malonate, 0.05M, pH 6.9-7.6. Periods of washing 2-308 hours.

(ii) *Respiratory Quotients; pH* 7

These were measured by the two-vessel method of Warburg. The reliability of the method for carrot tissue, when the pH is high, is discussed by Turner and Hanly (1949). In those experiments in which the pH of the malonate was 7.0 the two-vessel method is inaccurate owing to the presence of bicarbonate in the solution: the results were therefore corrected by using

several supplementary vessels in which the carbon dioxide retention was measured by acid tipping.

In contrast to the high R.Q. in malonate at pH 4-4.5, the R.Q. of tissue respiring in neutral potassium malonate solution is below unity during the early stages of the experiment. The extent of the "bicarbonate" error and the true change in R.Q. with time are illustrated in Table 4; accompanying the R.Q. change there is a fall in pH of the medium from 7 to about 6.

TABLE 4

RESPIRATORY QUOTIENTS IN NEUTRAL MALONATE SOLUTIONS

Malonate solution, initial pH 7.3, causing stimulation of $Q_{O_2}^{FW}$	
R.Q. over first 200 min. after tipping	0.68 Direct method
	0.76 Corrected by acid tip
R.Q. over second 200 min.	0.98 Corrected
R.Q. over whole period (400 min.)	0.75 Direct method
	0.85 Corrected
Final pH, in vessels with potash absorbent 6.2	

As far as can be ascertained in short manometric experiments, the corrected R.Q. following malonate addition at pH 7 is significantly below unity (0.8-0.9) for some one to two hours, after which it drifts upwards to a value of unity or even slightly above this. We have no R.Q. figures for malonate effects at pH 5 and 6.

(iii) *Reversibility; pH* 5-7

Only a few experiments were made to discover the effect of replacing the malonate solutions by water; the transfer was always made two hours after malonate addition. At pH 5 and 6 there was a rapid recovery of the $Q_{O_2}^{FW}$ from the depressed level (self reversal not being complete) to levels above that normal for water. At pH 7 the $Q_{O_2}^{FW}$ was increased slightly above the level reached previous to malonate stimulation.

(*d*) *Discussion*

(i) *Inhibition at pH* 4-4.5.—Malonic acid is clearly effective as an inhibitor of carrot root respiration only at the lowest pH experimentally tested, viz. 4-4.5. Solutions at this pH, of concentration 0.03-0.05M, usually bring the oxygen uptake down to a steady state (the "ground or basal respiration"). Lack of recognition of the importance of pH is almost certainly responsible for early statements that plant respiration is not inhibited by malonate.

The simplest hypothesis to explain the pH effect on inhibition is that the malonic acid inhibits the enzymic activity only when it is in the un-ionized form. Clear evidence of this might be provided by experiments of the kind described for azide by Stannard and Horecker (1948). Unfortunately at low

concentrations of malonate at pH 4 and higher concentrations at pH 5 and 6 the effect produced by malonate is complex (depression followed by recovery and concomitant change of R.Q.). The form of any curve plotting percentage inhibition with pH will vary with the total concentration, the age of the disc, and the time at which percentage inhibition is determined.

Stannard and Horecker (1948) found that azide was more effective as a respiratory inhibitor in frog muscle at low pH. They also obtained a similar pH effect with extracted enzyme systems and they showed that both with cyanide and azide the cytochrome-oxidase-inhibitor complex is formed only with the undissociated acids HN_3 and HCN. *In vitro,* succinic dehydrogenase from the carrot root is hardly active at pH 4 but at least the evidence suggests that since malonate is fully effective as the enzyme inhibitor at pH 7, it must act as an ion.

If, then, we assume (with Potter and Dubois 1943) that one of the malonate ions is the effective inhibitor, we still have to explain the marked pH effect on inhibition *in vivo.* Bonner published a single curve of inhibition *versus* pH for one concentration of malonate and concludes that because this "closely follows the titration curve of malonate the pH effect is due to the penetration only of HM′." Other workers on cellular inhibitors (e.g. Thimann and Schneider 1938; Simon and Blackman 1949) assume that the cell possesses an external membrane relatively impermeable to ions but permeable to molecules. The argument would then be that malonate enters the cell only as the molecule and dissociates within the cytoplasm to give the reactive ion. Although there is good evidence for such differential permeability for the tonoplast, there are ample reasons for doubting that the cytoplasm is much more permeable to uncharged than to charged particles. Moreover, the internal equilibrium concentration of malonate ions would not be determined by the ease of entry of molecules, but by the Donnan effect.

Robertson and Wilkins (Robertson 1951) have shown that the entry of anions from KCl is greater at low pH. They explain this as due to the suppression of ionization of weak electrolytes (which provide indiffusible ions in the cytoplasm) by H^+ ions from the medium. According to the Donnan effect, this would allow greater entry of anions from the medium. Such an explanation would of course apply also for malonate and with such a weak acid the pH effect would be enhanced because at pH 4 the external concentration of HM′ is maximal (Fig. 1). Various complicating factors may well be concerned, however, e.g.:

(i) Loss of inhibitor ions from cytoplasm to vacuole;

(ii) Exchange of K^+ in medium for H^+, tending to increase the internal pH;

(iii) Buffering of the internal pH by the system sugar $\rightleftharpoons$ acids as shown by Ulrich (1941);

(iv) Reversal of inhibition by malonate if acids of the Krebs cycle are produced in (iii).

With such a complex system, it seems impossible to interpret the pH effect on inhibition in any simple fashion.

The rapid recovery after washing is in accord with the view that malonate forms a dissociable complex with succinic dehydrogenase; the increased $Q_{O_2}^{FW}$ after washing away the malonate suggests that during the period of inhibition some intermediate substrate accumulates which can rapidly be metabolized only in the absence of malonate. The R.Q. data support this view. During the inhibition of respiration the R.Q. is *c.* 3 and fermentation is occurring. After washing, the R.Q. falls rapidly but it nevertheless remains above unity (*c.* 1.3) even after respiration has risen above the normal level. Such a high R.Q. and stimulation of respiration would be the accompaniment of organic acid metabolism; accumulation of succinate in the presence of malonate has been established for some tissues, but up to the present we have been unable to demonstrate it for carrot tissue.

(ii) *Stimulation at pH* 7.—In complete contrast to its effect at pH 4, malonate at pH 7 causes an initial stimulation of oxygen uptake by carrot root tissue. Unlike the similar stimulation brought about by KCl, this is accompanied by a low R.Q. of 0.8-0.9 and it is followed by a slow downward drift of the respiration to or below the control value in water, the R.Q. rising concurrently to unity or more. Burris and Wilson (1939) suggested that malonate may be respired at pH 7, but there is no direct evidence for this. We prefer to adopt the hypothesis that at pH 7 the internal concentration of malonic ions is kept low and that the stimulation of respiration is connected with the presence of K^+ in high concentration in the medium (Fig. 1). We presume a ready exchange of K^+ for H^+ and a tendency for the internal pH to rise (in our experiments the pH of the external solution consistently decreased to about 6, but this was no doubt in part due to carbon dioxide retention). According to Ulrich (1941) the internal pH rise due to cationic exchange is countered by the formation of organic acids (cf. according to Burstrom (1940) by malic acid). Such a process could well lead to high oxygen uptake and a low R.Q., while increased organic acid would also be expected to reduce malonate inhibition by competition.

The fall in pH of the external solution from 7 to 6 during these experiments provides us with a possible explanation of the fall in respiration rate which succeeds the initial rise, as at pH 6 malonate normally brings about partial inhibition.

An alternative explanation of the stimulation caused by malonate at pH 7 is that the salt acts in the same way as KCl, which stimulates respiration and is accumulated in the vacuole. A similar suggestion has already been made for succinate (Turner and Hanly 1949); it is not inconceivable that an ion might be transported across the cytoplasm by a carrier and thus be unable to play its part as an inhibitor. The concept of "salt stimulation" by malonate would, however, be more acceptable if it could be shown that malonate accumulates in the vacuole.

(iii) *Partial Inhibition, Followed by Recovery.*—Partial and temporary inhibition of the malonate-sensitive fraction of the respiration is induced either

by sub-optimal concentrations (0.01M) of malonate at pH 4.0 or by higher concentrations (0.05M) at pH 5 or 6 (Table 5). In such solutions the oxygen uptake is partially inhibited ($Q_{O_2}^{FW}$ 40-80); the inhibition is not maintained, but the rate rises until complete recovery to the normal rate is effected, usually within three hours. In Table 5, solution *a* causes maximal inhibition to the basal level, while *b* and *c* produce the partial temporary inhibition described above. One possibility, already discussed, is that solutions *b* and *c* act alike because both provide low concentrations of malonic molecule. It is more likely, however, that the tendency in *b* for the low pH to favour penetration of HM′ is counterbalanced by the low concentration of HM′, whereas in *c* the higher pH is associated with higher [HM′].

TABLE 5
IONIZATION AND MALONATE EFFECTS

	pH	Malonate Added	$[H_2M]$ $\times 10^{-4}$M	[HM′] $\times 10^{-3}$M	[M″] $\times 10^{-4}$M	Type of Inhibition
a	4.0	0.05M	30	46	10	Complete to basal level
b	4.0	0.01M	6	9	2	Partial and temporary
c	5.0	0.05M	3	40	87	Partial and temporary

For solutions of the type here concerned, the rate and degree of initial inhibition by a given concentration increase as the pH is altered from 7 to 4. (This is so at least for discs of one particular age.) The characteristic feature, however, is what may be termed self recovery from inhibition. This has also been reported for sub-optimal strengths of malonate with other tissues; for the carrot it only occurs when the inhibition of the malonate-sensitive respiration is incomplete. After such reversal to the normal rate the respiration may be again inhibited to the ground rate by the addition of cyanide, and hence we presume that recovery is probably due to reversal of the enzyme inhibition and recovery of the system operated by cytochrome and succinic dehydrogenase, previously inhibited by malonate at the succinic dehydrogenase enzyme.

It has been shown that added succinate can, under certain circumstances, reverse malonate inhibition and the most likely explanation of self reversal is the formation of succinate in concentration sufficient to compete with malonate for the enzyme. We have not been able to demonstrate accumulation of succinic acid in tissues in which inhibition to the basal level is complete; but it is possible that when inhibition is incomplete and only succinic dehydrogenase is inhibited, accumulation of succinic acid by oxidative formation may be sufficient to reverse the malonate inhibition. This is the explanation for self reversal given by Laties (1949*a*). Reversal may well be accelerated if the "Ulrich" effect noted above is operating to increase the concentration of organic acids.

The malonate effects at pH 7 and 4 are fairly uniform for discs of very varied age from cutting. The effects at pH 6, however, vary markedly with the "age" of the discs, as is shown in Figure 11; also by comparing Figures 12 and 13. For example, at pH 6.0, malonate applied to freshly cut tissue produced a clear-cut but partial inhibition of the malonate-sensitive respiration; in tissue washed for 300 hours the malonate caused stimulation; while for tissue washed for only about 200 hours, the malonate had no effect on the value of oxygen uptake. These results are possibly connected with changes in the nature of the permeability and nutritional status of the cells during aging and they may eventually throw some light on the nature of the so-called wound respiration in tissue slices.

(iv) *The Krebs Cycle.*—Although proof of the existence of a Krebs cycle in carrot is lacking, the facts described above fit well with the view that malonate at low pH inhibits part of the respiration of carrot tissue by inhibiting the succinic dehydrogenase link in such a cycle. Succinic acid is metabolized by this tissue and succinic dehydrogenase has been obtained from it and shown to possess many of the characteristics of the animal enzyme — for instance, it is competitively inhibited by malonate. The inhibition *in vivo* may be removed by washing away the malonate and this is in accord with the view that malonate forms a dissociable complex with succinic dehydrogenase. Moreover, we present evidence for the reversal of the inhibition by addition of succinate. Our earlier failure to obtain reversal (cf. also Machlis 1944) was clearly due to the use of too high a concentration of malonate. In view of what is known about the effect of malonate on other enzymes of the Krebs cycle, it appears probable that, for carrot tissue at least, when concentrations greater than 0.04M are applied, the cycle is interrupted at more than one place (although the same fraction of the respiration is inhibited). Hence reversal by the addition of succinate alone would not be expected.

The evidence for the operation of a Krebs cycle in carrot tissue would be stronger if it could be shown that application of malonate at the pH that allows respiratory inhibition also brings about the accumulation of succinate, oxidatively, in the tissue. So far our experiments have failed to show such accumulation. We suggest that such accumulation does, in fact, begin when the actual inhibitor concentration in the cell is low, but that it cannot then be demonstrated because it leads to reversal of inhibition. In tissue in which inhibition of respiration is complete to the basal level, we take it as highly probable that the inhibitor concentration is such that not only S.D., but also other enzymes of the Krebs cycle are put out of action. Accumulation of succinate when the S.D. is inhibited is only possible given a continual supply of oxaloacetate and pyruvate. We have no reason to think that oxaloacetate could not be supplied in such tissue from such a source as aspartic acid, but if its oxidation is inhibited by high concentration of malonate, then succinate accumulation could not occur. Under such conditions one would expect the pyruvate from glycolysis to be diverted to give fermentation products and it is

significant that in the carrot tissue in which succinate accumulation is not demonstrable, the R.Q. accompanying malonate inhibition is very high (*c.* 3). On the other hand, for tissues in which succinate accumulation does occur, much lower R.Q.'s have been recorded; calculation shows that the R.Q. of succinate formation would be less than 1 if the necessary oxaloacetate came by carboxylation of pyruvate; 1.5 if it came from a pre-existent store in the cells; and again less than this if aspartic acid were the source.

(v) *Basal Respiration.*—In many plant tissues, respiration is not reduced to zero by cyanide or malonate; in carrot tissue the curve relating respiration to the concentration of either of these inhibitors approaches an asymptote at a rate of the order of $Q_{O_2}^{FW} = 30\text{-}40$, although it is true that with further increase in the inhibitor concentration, the curve takes a downward course again (Fig. 2). This latter effect may be ascribed to secondary inhibitions and the view is put forward that in carrot tissue, as in some other plants, there is a basal respiration mediated by enzyme systems resistant to cyanide, malonate, and carbon monoxide.

The autotrophic respiration of *Chlorella* is stated to be completely cyanide-resistant (Genevois 1927), and James and Beevers (1950) report that the respiration of *Arum* spadix tissue is not inhibited either by cyanide or by malonate at pH 3.5. Commoner (1940) has reviewed the evidence for a cyanide-resistant basal respiration in other tissues. More recently Warburg, using as evidence the hyperbolic nature of the curve "per cent. inhibitor/[CN]'," states categorically that inhibition by cyanide is as complete as is required by the dissociation law. But Warburg has ignored Commoner's argument concerning the danger of expressing inhibition in terms of percentage of the control, while, for the tissues he quotes, the basal respiration, if existent, may be of a very low order. As noted above, we find that the effects of malonate on carrot respiration are closely similar to those brought about by cyanide and carbon monoxide. The basal rates attained in all three inhibitors are of the same order; other evidence (Robertson and Turner 1945; Weeks and Robertson 1950) is available that supports the view that the cyanide- and CO-sensitive factor of the respiration is mediated by the cytochrome oxidase system, and that salt accumulation is not possible if this fraction of the respiration is inhibited. We take it that malonate, acting on the succinic dehydrogenase linked to the cytochrome oxidase system, also inhibits the cyanide-sensitive system and a *prima facie* case is made out for the existence of a basal respirator. Investigation of the enzyme systems (e.g. of flavoprotein type) concerned in such basal respiration is long overdue.

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